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This project focuses on angiogenesis, the growth of new blood capillaries, as a potential target for breast cancer therapy and diagnosis. Our approach is based on our past demonstration that many of the signal transducing molecules that mediate the effects of angiogenic mitogens and extracellular matrix molecules are concentrated within specialized cell adhesion sites, known as the focal adhesion complexes (FACs). Over the past year, we demonstrated that a known angiogenesis inhibitor, TNP-470, inhibits the activity of $_{pp}125^{FAK}$, a tyrosine protein kinase that is also a major structural component of the FAC. We also began to develop and screen monoclonal antibodies using isolated FACs as an immunogen. Production of these antibodies and identification of molecules that mediate growth modulation in capillary cells should facilitate development of new angiogenesis inhibitors as well as alternative methods for breast cancer diagnosis.

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# INTRODUCTION

This project is based on the concept that human breast cancer growth may be suppressed by inhibiting the growth of tumor blood vessels, or what is known as angiogenesis, rather than by targeting the tumor cells themselves. This concept is based on the observation that the cells of solid tumors require continual capillary ingrowth for their own growth and expansion (1). Importantly, while capillary endothelial (CE) cells in tumor microvessels grow very rapidly, normal CE cells are usually quiescent. This differential in CE cell turnover rates therefore provides a potential therapeutic window to selectively prevent tumor expansion without producing generalized cytotoxicity.

Part of the "proof of principle" for this type of approach, at least in animals, is based on our discovery of the potent angiogenesis inhibitor, TNP-470 (also known as AGM-1470). TNP-470 inhibits the growth and metastasis of many solid tumors in animal models, including breast cancers (2,3). Because this compound does not act by killing cells (it only prevents quiescent CE cells from growing and migrating), it lacks the systemic toxicity that characterizes conventional anti-cancer treatments. When combined with cytotoxic agents (e.g., cytoxan), TNP-470 actually induces complete tumor regression in a large percentage of animals (4). TNP-470 entered Phase I clinical trials in 1992 and it is currently being tested in Phase II clinical trials for treatment of a variety of solid human tumors, including breast cancer. The first complete clinical response (tumor regression) in the absence of obvious toxicity was reported in an abstract form last spring (5).

TNP-470 is an exciting prototype angiogenesis inhibitor with great potential for preventing tumor formation and metastasis as well as treating existing tumors. However, it is likely that it will be possible to develop even more potent and less toxic angiogenesis inhibitors in the future. The major limitation that we face is that the molecular basis of TNP-470 action remains unknown. Thus, one of the major objectives of our proposal is to identify molecular targets that mediate angiogenesis inhibition induced by TNP-470. We also proposed to identify proteins that mediate growth activation by angiogenic mitogens. While the former molecules may represent potential targets for therapeutic intervention, the latter could be used as molecular markers for breast cancer diagnosis.

Our experimental design is based on past work from our laboratory which revealed that many of the signal transducing molecules that mediate the CE cell growth activation are immobilized on the cytosketon at the site of integrin receptor binding within a specialized adhesion structure that is known as the focal adhesion complex (FAC) (6). In addition to integrating signals elicited by binding of growth factors and extracellular matrix molecules, the FAC also provides a path for transfer of mechanical forces across the cell membrane and to the cytoskeleton (7). Mechanical forces transferred across integrins in the FAC drive changes in cytoskeletal organization and cell shape that are required for cell cycle progression (8,9). We recently developed a method to physically isolate intact FACs away from the remainder of the cell and cytoskeleton and have used this approach to demonstrate that isolated FACs retain multiple signal transducing activities in vitro (e.g., protein tyrosine and inositol lipid kinase activities; 6). Our initial application also included data which suggested that the FAC also may play an important role in angiogenesis inhibition. Specifically, we had preliminary results which suggestedthat TNP-470 alters tyrosine phosphorylation of a subset of FAC proteins.

Thus, the GENERAL GOAL of our initial proposal was to identify putative molecular targets which could mediate growth stimulation by soluble mitogens or angiogenesis inhibition by TNP-470 in CE cells. The more specific objective was to develop monoclonal antibodies directed against these FAC proteins in order to isolate, sequence, and clone these potential regulatory molecules. Once identified, we would be in a position to determine their role in capillary growth control and thus, to establish a more rational basis for drug design in the field of angiogenesis inhibition. Production of antibodies that recognize growth-associated antigens in CE cells also might form the basis for a more quantitative diagnostic assay for use with tumor biopsy materials.

The specific TASKS proposed under the STATEMENT OF WORK of this grant are:

1. To develop monoclonal antibodies against FAC molecules that are preferentially expressed in growth-stimulated CE cells.

2. To identify antibodies that recognize FAC proteins whose phosphorylation state appears to change in response to treatment with angiogenesis inhibitors.

3. To construct human breast CE cell λgt11 cDNA expression libraries and screen them with these monoclonal antibodies to isolate cDNA clones for the relevant FAC proteins.

4. To explore whether these monoclonal antibodies preferentially detect angiogenic microdomains in histological sections of human breast cancers.

## BODY OF THE REPORT

TASK 1:

The major objective of this task is to develop monoclonal antibodies directed against FAC proteins from growth-stimulated CE cells. During the past year, intact FACs were isolated from basic fibroblast growth factor (FGF)-stimulated CE cells using magnetic microbeads coated with synthetic RGD-containing peptide, as previously described (6). Approximately 200 ug of FAC protein was used to immunize two six week old female Balb/cJ mice; the protein was emulsified in complete Freund's adjuvant and injected into the peritoneum. Two, four and six weeks after the initial antigen challenge, equal amounts of immunogen mixed with incomplete Freund's adjuvant were injected in a similar location; a final intravenous boost was administered two weeks later. Four days after the final boost, the spleen was removed and the splenocytes were isolated and fused with myeloma line P3/NS1/1-Ag-1cells using PEG4000 using standard protocols (10). Hybridomas were grown and selected in DMEM/HAT medium for a period of two weeks. A total of 29 hybridoma cell lines were generated as a result of this first fusion. Once cell clones became visible and the medium turned acidic, small aliquots of hybridoma supernatant medium was removed for testing. Simultaneously, cell lines were expanded and frozen to preserve their original genetic integrity.

We have explored multiple screening protocols over the past year in an attempt to expedite antibody development:

•*Western blotting.* The hybridoma supernatants were first screened to determine if they recognized denatured cytoskeletal (CSK) proteins in Western blots (the FAC represents a subset of the CSK fraction). CSK-associated proteins were isolated from FGF-stimulated subconfluent BCE cell cultures by extracting the cells with an ice cold CSK stabilization buffer containing 0.5% Triton-X-100 and then dissolving the insoluble CSK framework in SDS sample buffer, as previously described (6). This CSK preparation was separated by SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blot analysis. A casette miniblotter was used to simultaneously test multiple (up to 56) hybridoma supernatant fractions as well as positive controls (commercial monoclonal antibodies directed against known FAC proteins). Positively stained bands were identified using affinity-purified horse radish peroxidase-conjugated anti-mouse IgG and a chemiluminescence visualization system (Amersham). Using this approach, several of the hybridoma supernatants have been found to label specific CSK protein bands (Fig. 1). Hybridoma lines coded as # 6, 17, & 18, exhibited single positive bands that migrated with apparent molecular weights of 110, 65, 65, and 100 Kd, respectively. Hybridoma line # 24 had two positive bands (105 & 138 Kd); # 12 exhibited several positively stained bands with various molecular weights (49, 57, 107 Kd). Control experiments carried out in parallel using commercial monoclonal antibodies specific for two different FAC proteins,

paxillin (68 Kd) and vinculin (115 Kd), all labelled specific bands with appropriate molecular weight, confirming the validity of this approach.

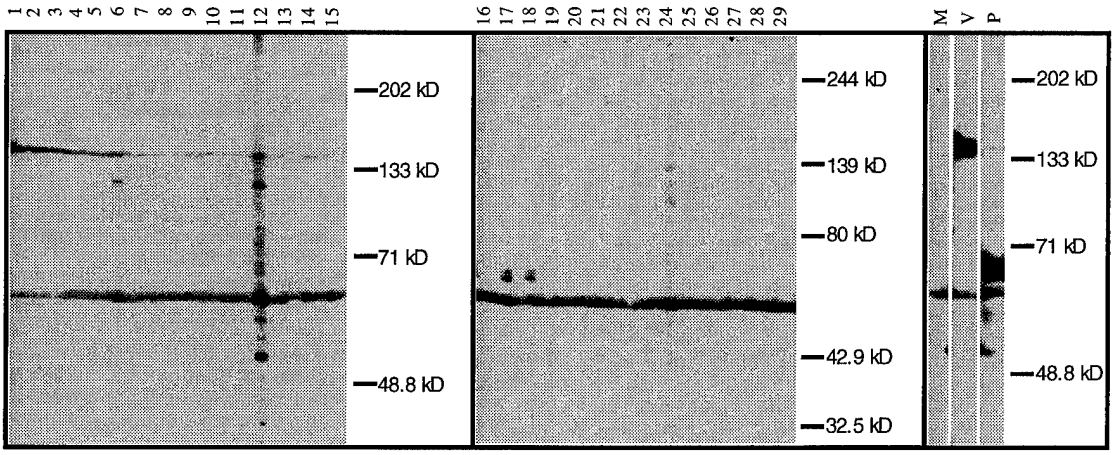


Fig. 1. Western blot screening. SDS-PAGE of the detergent-insoluble (CSK) fraction from FGF-stimulated subconfluent BCE cells . Equal amounts of CSK fraction were separated by SDS-PAGE (7.5% gel), electrotransferred to nitrocellulose membranes, and stained with the initial 29 hybridoma line supernatants (coded from 1 to 29). Positive controls included use of commercial antibodies directed against paxillin and vinculin (respectively lanes P and V) in parallel lanes. Hybridoma growth medium (M) was used as a negative control. Non-specific staining bands were observed in the range of 59 and 125 - 135 Kd in multiple specimens in these studies. We are currently trying to optimize conditions to minimize staining of these bands.

•*Solid-phase immunoassay.* Western blots allowed us to detect antibodies that recognize SDS-denatured antigens. To screen for antibodies that recognize FAC proteins in more native, folded conformation, we have begun to develop solid-phase immunoassays, using either whole cells or intact CSK preparations. Confluent CE cell monolayers were trypsinized and replated on gelatin-coated 96-well plates. After 6 hours, cells were either fixed with 3.7% paraformaldehyde (PFA) and shortly permeabilized with 0.2% Triton-X-100 (Whole Cell Fraction) or first extracted in CSK stabilization buffer containing Triton-X-100 and then fixed in PFA (CSK fraction). After blocking non-specific sites with BSA, hybridoma supernatants or commercial antibodies directed against known FAC proteins were incubated with these solid phase substrates (i.e., whole cells or CSK preparations). Subsequent incubation with affinity purified biotinylated anti-mouse IgG antibodies, avidin-biotinylated alkaline phosphatase complex and appropriate substrate (p-nitrophenyl phosphate) yield a colored product that was measured spectrophotometrically at 405nm.

The assay was initially optimized for sensitivity using monoclonal antibodies directed against vinculin, talin, and paxillin. The assay was optimized in terms of the number of cells needed to detect a positive signal, fixation conditions, and secondary antibody concentration. We also carried out appropiate negative controls (e.g., in the absence of primary antibodies, secondary antibodies, or enzymes) and comparable results were obtained with several enzymes (i.e., horse radish peroxidase vs alkaline phosphatase). Using the optimized assay (described above), four hybridoma supernatants (cell lines # 6, 7, 12 & 24) were found to be positive using either whole cells and CSK preparations as the source of solid-phase antigen (Fig. 2). Interestingly, hybridoma lines 6, 12 and 24 were also positive by Western blotting (Fig. 1), while the supernatant from line 7 did not appeared to recognize the antigen after exposure to SDS.

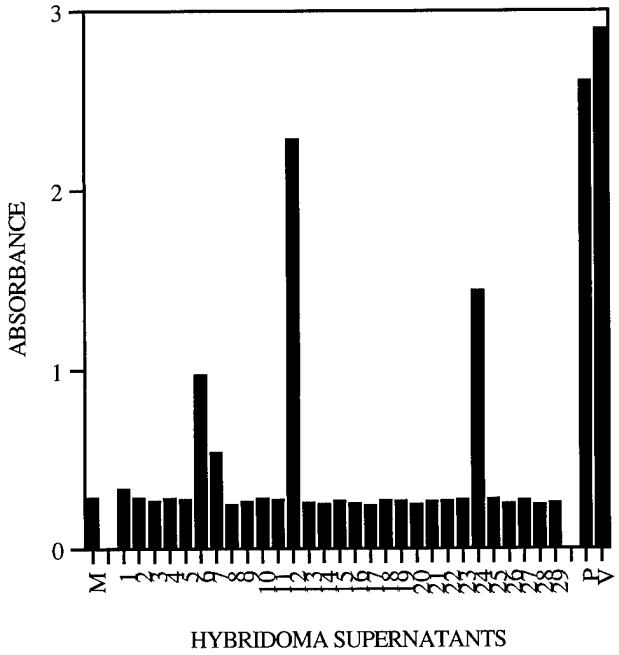


Fig. 2. Solid-phase immunoscreening. Supernatants from hybridomas coded 1 to 29 were added to adherent CE cells after fixation in PFA treatment followed by detergent permeabilization. Bound antibodies were detected with biotinylated anti-mouse IgG antibodies and avidin-alkaline phosphatase complexes. Commercial anti-paxillin (P) and anti-vinculin (V) monoclonal antibodies were used as positive controls; hybridoma growth medium (M) was used as negative control.

•*Immunfluorescence Detection.* We are currently developing another screening assay that relies on immunofluorescence staining to expedite detection of FAC-associated antigens in both growth factor-stimulated and TNP-470-inhibited CE cells. FAC proteins commonly appear as small, spear-like streaks at the base of the cell when analyzed by immunofluorescence microscopy. However, this form of identification is very subjective and it is often difficult to be sure that staining is specific or positive. The characteristically large variability in FAC size, shape, and distribution would therefore make it difficult to use FAC staining as a screening method. However, we have overcome this problem by adapting a recently described micropatterning technique to create culture surfaces that contain regularly spaced, matrix-coated adhesive islands on the size of individual FACs (Fig. 3). This work, which was carried out in collaboration with George Whitesides (Harvard, Dept. of Chemistry), involves microcontact printing of self-assembled monolayers of alkanethiols on gold transparently layered onto glass coverslips (9,11). A hydrophobic methyl-terminated thiol was used to pattern circular SAM regions (3-5 um diameter with 6 to 10 um spacing) that adsorb fibronectin. The surrounding non-adhesive regions were coated with a tri-ethylene glycol terminated thiol that prevents protein adsorption. When CE cells are plated on these surfaces they only attach to the fibronectin-coated islands and thus, they form FACs (e.g., as visualized by positive vinculin staining) that exhibit the same size, shape and spacing as that characteristic of the micropatterned substrate on which they adhere (Fig. 3). Due to the regularity of this pattern, we believe that this staining approach may be used as a new screening assay for antibodies that recognize FAC proteins, a method that could be automated in the future. To facilitate these studies, we have designed and fabricated a holding device that will allow us to simultaneously screen 20 different hybridoma supernatants on a single micropatterned coverslip (Fig.4). Similar screening assays can then be carried out with cells cultured in the presence of growth stimulators (bFGF) or inhibitors (TNP-470) to identify antibodies that recognize growth state-specific FAC components.

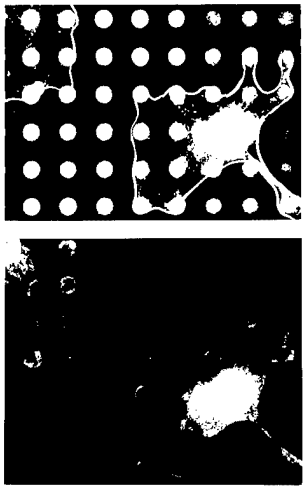

Fig. 3. Immunofluorescence views of CE cells adherent to micropatterned surfaces coated with circular adhesive islands (5 um diameter) coated with fibronectin. Top) CE cells spread over multiple islands which stained positively using antibodies to fibronectin. Cell bodies are outlined in white. Bottom) The same cells stained with antibodies directed against vinculin. Note the regular spacing, shape, and size of the positively stained circular FACs.

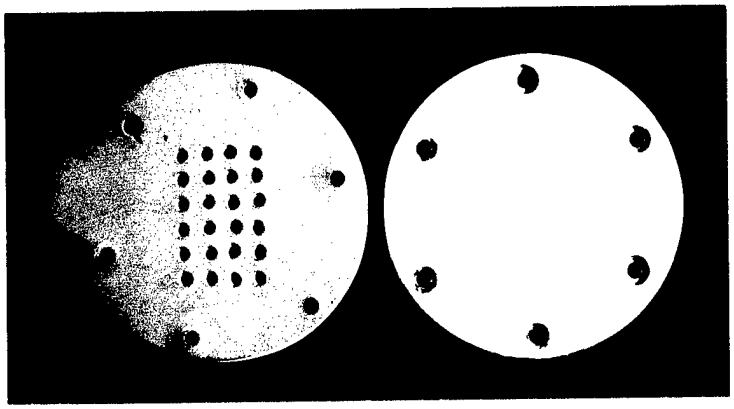

Fig. 4. Device designed for simultaneous immunostaining-based screening of multiple hybridoma supernatants using CE cells adherent to a single micropatterned surface. The micropatterned coverslip is clamped tight between the two drilled teflon plates. The use of a rubber gasket prevents the crosspilling of medium or cells between wells and thus, permits simultaneous staining of a single micropatterned coverslip using different antibodies or hybridoma supernatants.

TASK 2:

Over the past year, we also have attempted to complete our ongoing studies that were designed to more fully characterize the signal transducing activities of the FACs we isolate from CE cells. These are the same preparations that were used as an antigen in the hybridoma experiments. For example, we showed that FACs are significantly enriched for phosphatidylinositol phosphate kinase activity when compared to the remainder of the CSK (12). We also extended our studies on TNP-470 to determine if we could identify a specific target for this drug within the FAC. The characterization of the composition and signaling capabilities of isolated FACs was fully described in last year's Progress

Report and in recent publications (Plopper et al. *Mol. Biol. Cell* 1995, 6:1349-1365; McNamee et al., *Exp. Cell Res.* 1996, 224:116-122 ). The advances we have made in terms of defining the molecular basis of action of TNP-470 within the FAC are currently being written up for publication and thus, these results are summarized below.

In the last Progress Report, we presented data which suggested that the angiogenesis inhibitor, TNP-470, might alter CE cell growth by altering signaling through the FAC. These data included demonstration that TNP-470 alters the mechanical properties of the FAC (as measured using magnetic twisting cytometry; 7) and that it changes the tyrosine phosphorylation state of a doublet of FAC proteins migrating with apparent molecular weight of approximately 120 to 130 Kd. Importantly, the dose-response of these effects (max dose = 1 ng/ml) and their time course (effects visible with 30 min to 4 hr) correspond well with the inhibitory effects of TNP-470 on CE cell migration. Furthermore, these effects could not be detected by conventional biochemical analysis of whole cells using Western blots with anti-phosphotyrosine antibodies. Thus, the FAC isolation procedure appeared to offer an exciting handle with which to approach this problem.

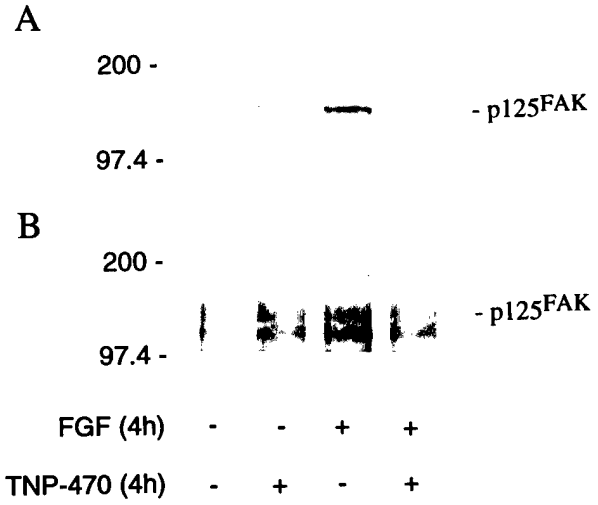


Fig. 5. Effect of TNP-470 on $_{pp}125^{FAK}$ tyrosine phosphorylation in the whole cell lysates Quiescent cells were plated on fibronectin-coated dishes for 4 h and whole cell lysates were prepared by extracting cells with modified RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% deoxycholate, 0.1% sodium lauryl sulphate, 1% Triton-X-100, 20 ug/ml aprotinin, 1 ug/ml leupeptin, 1 ug/ml pepstatin, 0.1 mM AEBSF, 200 uM sodium orthovanadate, 20 mM b-glycerophosphate, 30 mM sodium pyrophosphate). $_{pp}125^{FAK}$ protein was immunoprecipitated using antibodies to $_{pp}125^{FAK}$ antibodies and blotted with antiphosphotyrosine antibodies (A) or with $_{pp}125^{FAK}$ antibodies (B).

Over the past year, we explored whether $_{pp}125^{FAK}$, a major protein tyrosine kinase of the FAC that exhibits a molecular weight in the 120-130Kda range could represent a target for TNP-470. This is an exciting possibility since this protein has been shown to represent a point of convergence between growth factor and integrin signaling systems (13). It also appears to play a major role in cell migration, a key component of the angiogenic response (14). CE cells were plated cells on fibronectin-coated dishes for 4 hr and $_{pp}125^{FAK}$ protein was immunoprecipitated from whole cell extracts. The immunoprecipitated proteins were then separated by SDS-PAGE and transferred to nitrocellulose paper for Western blot analysis using anti-phosphotyrosine antibodies to quantitate changes in $_{pp}125^{FAK}$ tyrosine phosphorylation (Fig. 5 A). A basal level of tyrosine phosphorylation of $_{pp}125^{FAK}$ was observed in CE cells adherent to fibronectin (lane 1) which increased approximately 2 fold upon addition of the angiogenic mitogen, basic FGF (lane 3). Cell attachment to fibronectin in

the presence of TNP-470 did not significantly alter the basal level of $_{pp}125^{FAK}$ phosphorylation (lane 2). In contrast, TNP-470 treatment completely abolished the increase in $_{pp}125^{FAK}$ phosphorylation normally induced in response to growth factor stimulation (lane 4 compared with lane 3). These changes were not due to differences in protein loading since similar amounts of $_{pp}125^{FAK}$ protein was detected when the same blots were reprobed with antibodies directed against $_{pp}125^{FAK}$ protein (Fig. 6B). Importantly, similar results were obtained when we analyzed FACs isolated from CE cells (i.e., rather than whole cell extracts): again, TNP-470 inhibited $_{pp}125^{FAK}$ phosphorylation without interfering with its recruitment to the FAC (not shown). Thus, these data suggest that TNP-470 may specifically prevent growth-factor induced activation of this kinase within the FAC. This is important because TNP-470 has little effect on baseline motility or growth whereas it prevents CE cells from responding to a wide variety of soluble mitogens and motility factors. Furthermore, it adds additional support to our proposed approach which centers on identification of potential therapeutic targets within the FAC.

TASKS 3 &4:

These tasks have not been initiated since we have not yet identified critical molecular targets. Once we complete the initial characterization of our first set of monoclonal antibodies, we may include human breast cancer specimens in the screen to determine whether these probes may be useful for identification of growing CE cells in histological preparations.

Additional studies carried over the past 12 months-

In addition to the accomplishments described above, we also have initiated a number of studies to facilitate the future success of our project. During the past 12 months, we have developed methods to carry out 2D gel electrophoresis and have begun to use this approach to analyze our FAC preparations. In the future, we will use this approach to compare FACs from growth factor-stimulated cells versus those from cells treated with TNP-470 to identify functional state-specific protein expression patterns. We also now have human CE cells standardly growing in the laboratory that can be used for our monoclonal screening protocols as well as a source of FAC antigen.

## CONCLUSIONS

The main objective of this grant is identify specific molecular components of the FAC that mediate CE cell growth stimulation or angiogenesis inhibition. Over the past year, we have made progress in both directions. First, we have raised and begun to characterize our first series of monoclonal antibodies using a FAC isolated from growth factor-stimulated CE cells as the source of antigen. Second, we identified a major component of the FAC signaling complex, $_{pp}125^{FAK}$, as a molecular target for the known angiogenesis inhibitor TNP-470. This is the earliest known molecular event that is induced by TNP-470 treatment in CE cells and thus, it provides an exciting handle with which to further analyze the mechanism of angiogenesis inhibition. By combining both approaches with additional characterization of the molecular composition of FACs isolated from growth stimulated or inhibited CE cells, we should be able to identify critical molecular mediators of angiogenic regulation. This approach should therefore enhance our ability to develop new angiostatic compounds. The monoclonal antibodies probes we develop may also be very useful for the diagnosis and prognosis of breast cancer as well as for following tumor responses in patients treated with angiostatic therapies.

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